Proposed title for the Ph. D. work: Comparative toxicity of silver nanoparticles in cancer and normal cells

Submitted by: Arindam Bandyopadhyay

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Supervisors: Prof. Ansuman Chattopadhyay

Prof. Shelley Bhattacharya

Department of Zoology

Visva-Bharati, Santiniketan

Introduction:

Silver is a white and shiny metallic element positioned 47th in the periodic chart with the chemical symbol Ag, short for 'argentums' [Seiler *et al.,* 1988]. There have been 2,000 years for human beings' discovery and use of silver, with applications including jewellery, utensils, monetary currency, dental alloy, photography and explosives [Dibrov *et al*., 2002]. Silver compounds and ions have been extensively used for both hygienic and healing purposes [Benn and Westerhoff, 2008]; however, over time, the use of silver compounds and ions as an anti-infection agent has faded due to the advent of antibiotics and other disinfectants. In present days, renewed interest has arisen in manufactured silver nanomaterials because of their unusually enhanced physicochemical and biological properties and activities compared to their bulk parent materials. It is estimated that of all the nanoparticles in consumer products, silver nanoparticle (AgNP) applications currently have the highest degree of commercialization [Tran *et al*., 2013]. It has been widely used in consumer products ranging from disinfecting medical devices and home appliances to water treatments [Tolaymat *et al*., 2010; Li *et al*., 2008; Silver and Phung, 1996].

Relevance of studying toxicity of silver nanoparticles:

AgNPs exhibit broad spectrum bactericidal and fungicidal activity that has made them extremely popular in a diverse range of consumer products.In medicines, silver and AgNPs have a wide application including skin ointments and creams to prevent infection of burns and open wounds. Medical devices and implants are also prepared with AgNP-coating. Inthetextile industry, silver-embedded fabrics are now used in sporting equipment. The other consumer products where AgNPs have wide usage are plastics, soaps, pastes, mouthwash, food packaging, kitchen utensils, water filters, refrigerators, washing machine and even dietary supplements [Yon and Lead, 2008; Cohen *et al*., 2007; Sondi and Salopek-Sondi, 2004]. This has made AgNPs the largest and fastest growing class of NPs in consumer products applications [Tran *et al*., 2013].However, there are ample reports, which indicate the toxicity of inorganic AgNPs inthebiological system [Renata *et al,* 2012].Thus, there is urgent need to find out a safe or less toxicAgNP for human application. Anticancer property of AgNPs has also been reported [Barua *et al.*, 2016; Rath *et al*., 2014; Barua *et al*., 2013]. Adequate evaluation of the action both at *in vitro* and *in vivo* levels is prerequisite prior

tousing such particles as anticancer agents. Therefore, the proposed study is highly relevant and the expected outcome will haveanimportant contribution to environmental and medical sciences.

Objectives:

The main objective of the proposed study is to find outrelatively less toxicAgNPs for their use in commercial and pharmacological industriesthrough the comparative study of theirtoxicitywhich aresynthesized via different methods suchasusingplant extracts, amino acids, and chemicals. To achieve the goal, the experiments will be focused on:

1. Determination of LC50 dose of AgNPs for cancer cells and human peripheral blood mononuclear cells (HPBMCs) as well as for zebrafish (*Danio rerio*).
2. Study of *in vitro* toxicity on cancer cells and HPBMCs.
3. Study of *in vivo* toxicity taking zebrafish (*Danio rerio*) as model and
4. Elucidation of molecular mechanisms of the toxic effects of AgNPs.

*In vitro*toxicity studies using Human Peripheral Blood Mononuclear Cells (HPBMCs):

Fresh blood will be drawn fromthe healthy human donor. HPBMCs will be isolated. For this purpose, blood will be diluted with equal volume ofPBS and layered over Histopaque followed by centrifugation. Thebuffy coat will be aspirated into PBS for centrifugation. Cells will bewashed thrice repeatingthis step. The rationale for using peripheral blood samples from healthy human volunteers is to reflect toxicity analysis in humans.

Determination of lethal concentration 50 (LC50) by Trypan blue dye exclusion assay:

The *in vitro* cytotoxic effects of AgNPs will be estimated by the Trypan blue dye exclusion. Cells will be counted using a hemocytometer, and the number of viable (opaque) and nonviable (stained blue) cells will be scored by direct observation under a bright-field microscope. The results will be subjected to probit analysis.

In the cytotoxicity assay, various aliquots of AgNPs will be added to the blood samples and incubated for 2 h. The viability of the lymphocytes will be recorded. The concentrations and percent viability data will be subjected to probit analysis using the probit model program and

LC50 of the test compound will be calculated. 1/10thconcentration of determined LC50 of AgNPs can be used as treatment concentration for further toxicity assays.

Cell proliferation study by MTT assay:

Isolated HPBMCs will be stimulated by Phytohaemagglutinin M (PHA) and cultured in RPMI-1640 media with 10% FBS along with antibiotics. After24 h, different concentration of AgNPswill be added in triplicate and cultured for another 48 h. Then MTT will be added and cell viability will be checked in a spectrophotometer. Percentage of viable cells will be calculated taking the viabilityof the untreated cells as 100%.The assay is based on the reduction of the MTT, a yellow tetrazole, to purple formazan by cellular mitochondrial dehydrogenases in living cells. Expansion in the number of viable cells resulted in an increase in the activity of the mitochondrial dehydrogenases, which leads to the increase in the amount of formazan dye formed. The formazan dye produced by viable cells can be quantified spectrophotometrically.

Apoptosis detection via Hoechst staining:

Nuclear fragmentation along with DNA degradation is the preliminary detecting sign of apoptosis induction. In this study cells will be fixed in 4% paraformaldehyde after treatment with different concentration of AgNPs. Cells will be placed onglass slides and dried in air, stained withHoechst and washedwith PBS. Stained cells will be examined underafluorescent microscope and analyzed for nuclear breakage.

Cell cycle analysis by FACS:

For flow cytometry,cells from both treated and untreated control will bemixed with phosphate buffered saline (PBS) and centrifuged. Then it will be resuspended in RBC lysis bufferandwill be kept in ice. Following another centrifugation, cells will bewashed, fixed, and permeated with ice-cold methanol. Then RNaseand propidium iodide per sample will be added and incubated in the dark. The samples will be analyzed by a flow cytometer and data acquisition and processing will be performed with particular software.

Cellular toxicity detection by different redox enzyme activity assays:

Both treated and untreated control HPBMCs will be assayed for various enzymatic activities for detecting cellular oxidative stress level.

Western blotting for apoptotic pathway detection:

Apoptosis or programmed cell death includes many pathways to take place atthecellular level. The apoptotic cascades include many proteins which are detectable by western blotting technique. Expression pattern of some apoptosis-related proteins like BAX, BCL2, Caspase 3 will be assessed by immunoblotting to evaluate apoptosis induced byAgNPsat different concentrations.

Chromosomal aberration studies to evaluate genotoxic effect:

HPBMCs will be cultured and metaphase plates will be prepared after treatment with different concentration of AgNPs to detect the genotoxic effect of the nanoparticles.

Sister chromatid exchange studies for evaluation of genotoxic effects:

Sister chromatid exchange (SCE) is the exchange of genetic material between two identical sister chromatids and is considered as a toxicological parameter. Study of sister chromatid exchange in both untreated control cells and cells treated with AgNPs can give an account of AgNP-induced genotoxicity. To address this issue heparinized blood will be taken, stimulated by phytohemagglutinin-M (PHA) along withtheaddition of BrdU and cultured in RPMI 1640 with 10% FBS along with antibiotics. After 24 h, the HPBMCs will be treated with AgNPs and will be cultured for another 48 h. Colcemid will be added prior to harvesting. After centrifugation and discarding the supernatant, fixative will be added and kept overnight. cell suspension will be prepared by repetitive centrifugation. Slides will be prepared by flame dry technique and incubated in dark with Hoechst stain. The slides will be kept in sunlight for 30 to 40 minutes. The slides will be studied underalight microscope after staining with Giemsa stain and mounting.

In vivo toxicity studies taking zebrafish (*Danio rerio*) as model:

For the present investigation adult zebrafish (*Danio rerio*) will be selected asthetest organism. Zebrafish is now widely used in many research areas due to several advantages like easy availability, low maintenance cost, and breeding in laboratory condition as well as similarity with human genome [Lawrence, 2007]. After collecting from local sources zebrafish will be allowed to acclimatize to laboratory condition for a minimum of 2 weeks prior to the experiments. It will be maintained at standard conditions (28 ± 1ºC temperature in 14 h of light:10 h of dark condition) in a 50 L tank containing continuously aerated water and will be fed twice daily with commercial fish-food.

Determination of LC50 of silver nanoparticles:

For the present study, the test fish will be exposed to AgNPs at various concentrations for 96h to determine the LC50. The zebrafish will then be exposed to $1/10^{th}$ – $1/20^{th}$ concentration of determined LC50 for 60 days. Subsequently on 7^{th}, 15^{th}, 30^{th} and 60^{th} day fish will be sacrificed and brain, liver and kidney will be collected for further toxicological assays as follows.

Histological studies:

For this purpose, tissues from both untreated control fish and fish exposed toaparticular concentration of AgNPs will be fixed in Bouin's fluid for 24 h. After fixation, the tissues will be dehydrated with alcohol up-gradation (70% <90% <100%) followed by ½ xylene and ½ absolute alcohol treatment. Dehydrated tissues will be further infiltered with paraffin and paraffin blocks will be prepared. Tissue in the block will be then microtomed to get sections of uniform thickness (5-6 µm) followed by proper section stretching on Mayer's albumin fixed glass slides.

For staining, slides with proper section stretching will be deparaffinised using xylene followed by hydration in descending grades of alcohol (100%> 90%> 70%> 50%> 30%). Hydrated tissue sections will be stained by Delafield hematoxyline and washing will be done using distilled water. The hematoxyline-stained tissue will be further passed through ascending grades of alcohol (30%> 50%> 70%> 90%> 100%) followed by eosin staining. The stained section will then be exposed to absolute alcohol and xylene. Finally, mounting will be done using DPX for microscopic analysis.

Biochemical assays:

At the end of the planned exposure, liver, brain and kidney from the fish will be collected. Then it will be homogenized in phosphate buffer and centrifuged to get the supernatant which will be used for experimental measurements.

Estimation of Reactive Oxygen Species (ROS), Glutathione (GSH) level and Malondialdehyde (MDA) content:

ROS levels in the fish tissue will be determined by the oxidation of 2′,7′-dichlorofluorescin diacetate (DCFDA) according to the procedure described by Contreras *et al.* (2005). DCFDA is a fluorogenic dye that measures ROS activity within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'–dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy.

The level of the GSH will be measured by the method of Akerboom and Sies (1981). Glutathione is a tri-peptide which exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent (H^++ e^-) to other unstable molecules, such as ROS. In donating an electron, glutathione itself becomes reactive but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress [Kenneth *et al.*, 1967]. In brief, to estimate the GSH content, supernatant will be added to a reaction mixture containing buffer, nicotinamide adenine dinucleotide phosphate (NADPH), 5-5'-dithiobis[2-nitrobenzoic acid] (DTNB) and glutathione reductase (GSH reductase). The general thiol reagent, DTNB, reacts with GSH to form the 412 nm chromophore, 5-thionitrobenzoic acid (TNB) which can be measured in a spectrophotometer against a sample blank.

The level of lipid peroxidation will also be determined. Lipid peroxidation refers to the oxidative degradation of lipids. In this process free radicals take electrons from the lipids (generally in cell membranes), resulting in cell damage. Quantification of lipid peroxidation is essential to assess oxidative stress in pathophysiological processes. Lipid peroxidation forms reactive aldehydes such as malondialdehyde (MDA) as natural bi-product. Measuring

the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. Briefly,thesample will be mixed with TBA–TCA–HCl mixture thoroughly and will be heated in a boiling water bath. The MDA in the sample reacts with thiobarbituric acid (TBA) to generate an MDA-TBA adduct. The MDA-TBA adduct can be quantified colorimetrically.

Assay of redox enzymes:

Glutathione-s-transferases (GSTs) activity will be determined following the method of Habig et al. (1974). GSTs are a group of enzymes that protect cells against toxicants by conjugating the thiol group of the GSH to electrophilic xenobiotics. For estimation of GSTs activity 1-chloro-2,4-dinitrobenzene (CDNB) will be used asasubstrate inthepresence of excess GSH. GST catalyzes the conjugation of GSH to CDNB through the thiol group of the glutathione. The reaction product, GS-DNB Conjugate, absorbs at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample.

Catalase (CAT) activity will be assayed following the procedure of Aebi (1984). CAT is an antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2), an ROS, to water and oxygen. For the estimation of enzymatic activity,thesample will be added to an assay buffer containing H_2O_2. In the assay, catalase will first react with H_2O_2 to produce water and oxygen, the unconverted H_2O_2 will react with a probe to produce a product, which can be measured colorimetrically at 570 nm. Catalase activity is reversely proportional to the signal.

Superoxide dismutase (SOD) activity will be assayed following the procedure of Ewing and Janero (1995). Superoxide dismutase (SOD) is one of the most important antioxidative enzymes. It catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The assay will utilize nitroblue tetrazolium (NBT) that will produce a water-soluble formazan dye upon reduction with superoxide anions. The superoxide anions, generated by xanthine oxidase (XO), is dismutated into H_2O_2 and O_2 by SOD. Thus, the rate of the reduction with a superoxide anion is linearly related to the XO activity and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method.

Detection of anti-oxidant gene expression by quantitative real-time PCR (Q-PCR):

Total RNA will be isolated from the tissues which will be quantified by absorbance at 260 nm usingaspectrophotometer. RNA will be reverse-transcribed into cDNA using reverse transcriptase. The gene-specific PCR primer pairs for *cat* and *gst* will be either designed or taken from published works [Liedtke et al., 2008] and Q-PCR will be performed.

Western blot analysisfor detection of the presence of Nrf2 and Keap1 proteins:

Western blotting will be done to detect the presence of Nrf2 and Keap1 proteins. Nrf2 is the master regulator of all cellular oxidation–antioxidation system at the transcriptional level [Lee and Johnson, 2004]. Under normal situation, Nrf2 remains bound to Keap1 [Kobayashi et al., 2004]. During oxidative stress, it dissociates from Keap1 and enters into the nucleus to bind to ARE (antioxidant responsive elements) and promotes genes responsible for anti-oxidation and phase-II detoxification system like cat, sod and gst [Li et al., 2011]. The assay utilizes specific antibodies to identify proteins that have been separated based on size by gel electrophoresis. The immunoassay will use a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel will be placed next to the membrane and application of an electrical current will induce the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

Keywords: Silver nanoparticles, anticancer, toxicity

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